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The Multiple Functions of Hemoglobin

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ABSTRACT: The aim of this review is to focus and discuss several parallel biological functions of hemoglobin besides its basic function of oxygen transport. In light of the information present in the literature the following possible physiological roles of hemoglobin are discussed: (1) hemoglobin as molecular heat transducer through its oxygenation-deoxygenation cycle, (2) hemoglobin as modulator of erythrocyte metabolism, (3) hemoglobin oxidation as an onset of erythrocyte senescence, (4) hemoglobin and its implication in genetic resistance to malaria, (5) enzymatic activities of hemoglobin and interactions with drugs, and (6) hemoglobin as source of physiological active catabolites.

KEY WORDS: hemoglobin, function.

I. INTRODUCTION

Large organisms have a high respiratory demand for oxygen that cannot be satisfied by diffusion of oxygen from the environment to the respiring tissues. Hence, they have been forced, in the evolutionary sense, to develop special systems to transport oxygen from the outer environment to the tis-

Symbols and abbreviations: Ald, aldolase; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; B3P, band-3 protein; BisTris, 1,3-bis-(tris-(hydroxymethyl)-(methylamino)-propane); CDB3, cytoplasmic domain of band-3 protein; 2,3-DPG, 2,3-diphospho-glycerate; F6P, fructose-6-phosphate; G, glucose; G3P, glyceraldehyde-3-phosphate; G3PD; glyceraldehyde-3-phosphate dehydrogenase; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; glutathione, γ-L-glutamyl-L-cisteinyl-glycine; cGMP, guanosine-3′,5′-cyclic-monophosphate; Hb, hemoglobin; HbA, adult human hemoglobin ($\alpha_2\beta_2$); HbA_{1c}, glycated adult human hemoglobin ($\alpha_2(\beta_{gly})_2$); HbA_2, A_2 hemoglobin ($\alpha_2\delta_2$); HbE, variant hemoglobin (α_2(β-26[B8]-glu→lys)$_2$); HbF, fetal hemoglobin ($\alpha_2\gamma_2$); HbS, sickling Hb, (α_2(β-6[A3]-glu→val)$_2$); HEPES, [4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid]; Ins-P_2, *myo*-inositol hexabisphosphate; LDH, lactate dehydrogenase; Met-Hb, met-hemoglobin (hemoglobin variously oxidized from one to four hemes); NADH, β-nicotinamide-adenine-dinucleotide; NADPH, β-nicotinamide-adenine-dinucleotide phosphate; Pyruv., pyruvate; PFK, phosphofructokinase; PK, pyruvate kinase; Tris, tris-(hydroxymethyl)-amino methane.

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sues, where it is utilized as the terminal acceptor in catabolism. These systems are represented by the so-called respiratory proteins — hemoglobins, erythrocruorins (i.e., invertebrate giant hemoglobins), hemocyanins, and hemerythrins — which differ greatly in the nature of the prosthetic group and the protein moiety (Brunori et al., 1977, 1980).

In humans, as in all vertebrates, the chemical basis for O_2 transport is represented by hemoglobin, which is packaged into specialized cells (erythrocytes) sufficiently pliable to withstand weeks of mechanical stress during their circulation in the cardiovascular system of the organism.

Because the uptake and delivery of oxygen is such a vital process, the hemoglobin molecule has been carefully engineered to display a fine tuning of its oxygen-carrying properties, characterized by the presence of both homo- and heterotropic interactions, in order to ensure an adequate delivery of oxygen in response to the physiological demands of the containing species. Hence, hemoglobin had, and still has, a major role in our progress toward understanding the detailed structural basis of allosteric regulation and continues to be the object of a great number of structural, dynamic, and engineering studies. We do not wish to review here these aspects, which are deeply discussed in many excellent papers and books (Perutz, 1980; Riggs, 1988; Weber and Jensen, 1988; Weber and Wells, 1989). In this article, we focus on several parallel biological functions that the hemoglobin molecule is known to display within the organism besides the basic function of oxygen transport and theoretically what we may call "biochemical economics". Thus, from the body of data already present in the literature, the emerging scheme is that of a mosaic of biological functions, which are centered on the oxygenation-deoxygenation cycle of Hb and whose relative importance is not easy to qualify. However, for the sake of clarity, we think it is useful to briefly describe the general mechanisms of hemoglobin modulation.

II. MODULATION OF HEMOGLOBIN FUNCTION: GENERAL CONCEPTS

Oxygen delivery at the level of the tissues is governed essentially by the molecular properties of hemoglobin and the physicochemical conditions under which it operates in *in vivo* conditions. In fact, oxygen binding by hemoglobin is controlled by two key molecular mechanisms, homo- and heterotropic interactions, which determine its oxygen affinity and its sensitivity to modulating cofactors.

Heme-heme or homotropic interactions are responsible for the sigmoid shape of the oxygenation curve, whose steep middle portion makes oxygen release suitably sensitive to small drops in oxygen pressure. Equally important are the environmental factors (hydrogen and chloride ions, carbon dioxide, and the intraerythrocytic organophosphates), whose interactions with the protein moiety (heterotropic interactions) control the position of the curve along the P_{O_2} axis or the overall oxygen affinity. In particular, an increase in the concentration of any of the aforementioned effectors lowers the affinity of hemoglobin, this change, of course, being reversible.

This fine tuning of the hemoglobin molecule is based on the principle of ligand-linked conformational change in a multisubunit structure. Hence, cooperative ligand binding arises from a coupling between the effects of ligand binding at the individual subunits and the interactions between subunits within the quaternary structure. In other words, the sigmoid shape of the binding

curve is a direct manifestation of the differences in Gibbs free energies for the successive oxygenation steps of the tetrameric molecule. This phenomenon is often described in terms of the two-states allosteric model proposed by Monod et al. (1965) and demonstrated by the structures of deoxy- and fully liganded hemoglobin as determined by X-ray crystallography (Shaanan, 1983; Fermi et al., 1984; Liddington et al., 1992). In such a mechanism, two alternative quaternary structures (low-affinity or T-state and high-affinity or R-state), having different affinities for oxygen, are in equilibrium with each other at all stages of saturation, and the binding of the ligand swings the equilibrium toward the high-affinity form (R-state). Within this description there is an oxygen concentration-dependent equilibrium between two distinct quaternary structures of the protein. At low oxygen concentration, when the hemoglobin is essentially deoxygenated, the low-affinity or T-state quaternary structure is favored. A progressive increase of the oxygen pressure, and hence of the degree of ligand saturation, shifts the equilibrium to the high-affinity or R-state quaternary structure. Hence, in the expression of cooperativity, the protein acts as a macromolecular transducer of free energy, as favorable increases in binding energy are compensated by parallel decreases in the free energies of conformational rearrangement within the protein itself.

The contemporaneous presence of alternative (at least two) structures is also at the basis of heterotropic interactions. In fact, the deoxy structure or T-state of Hb has a low affinity for oxygen and a high one for protons, chloride, organic phosphate, and carbon dioxide. In the oxy structure or R-state, these relative affinities are reversed. The fine tuning of Hb function brought about by heterotropic effectors is therefore based on the ability of these molecules to preferentially bind the low-O_2-affinity conformation, or T-state, and to stabilize this structure by forming salt bridges within or between subunits (Perutz, 1970). Hence, the unique allosteric properties of hemoglobin are such that protons, carbon dioxide, organic phosphates, and chloride ions all promote the release of oxygen.

Finally, we have to consider the effect of temperature, which is known to greatly affect the position of the oxygen dissociation curve. Thus, an increase in temperature of 10°C generally decreases the O_2 affinity from 1.5 to 2.5 times, depending on the experimental conditions. It may be worthwhile to recall that the shape of the oxygen dissociation curve of HbA appears to be independent of temperature over a large range of saturation, implying that the same amount of heat is released all along the saturation curve. In light of recent results (di Prisco et al., 1991), which have outlined the role of temperature in the unloading of oxygen at the tissues, heat absorption and release can be considered a physiologically relevant modulating factor, similar to hetero- and homotropic ligands.

III. HEMOGLOBIN AS MOLECULAR HEAT TRANSDUCER

Animal organisms have developed special systems to adjust to a wide range of environmental temperatures from about −40 to +50°C. The general mechanisms available for the stabilization of body temperature, dependent on the outer environment, are (1) generation of heat by the metabolic burning of food, (2) use of insulation and other devices to retain body heat, and (3) mechanisms for heat dissipation.

As far as the latter is concerned, recent papers (Giardina et al., 1993; Clementi et al., 1994) noted that, in some animal species and in the human fetus, hemoglobin may play a significant role in keeping constant the internal temperature of the containing organism through the thermodynamic characteristics of its oxygenation-deoxygenation cycle. Before going into the details of such mechanisms, it seems worthwhile to recall some important features linked to the temperature dependence of the reaction of hemoglobin with O_2. This is, of course, governed by the overall enthalpy change (ΔH), which in mammalian hemoglobins is generally exothermic, so that a decrease in temperature induces an increase of O_2 affinity.

In the case of a simple hemoprotein such as myoglobin, the heat released on O_2 binding is generally in the region of –60 KJ/mol, but for HbA this release of heat is reduced to about –35 KJ/mol because of the compensating effect of other O_2-linked processes; in this respect, it is informative to correlate the different contributions to the thermal effects measured when O_2 binds to hemoglobin. These may be summarized as: (1) intrinsic heat of oxygenation or the heat involved in the binding of O_2 to the heme iron, (2) heat of ionization of O_2-linked ionizable groups (Bohr groups), which is always endothermic (ΔH positive), (3) heat of O_2 solubilization (–12 KJ/mol; exothermic), (4) heat associated with the $T \rightarrow R$ allosteric transition, and (5) heat of binding of other ions, as organic phosphates and chloride.

In the case of HbA, ΔH is more exothermic at very alkaline pH values (pH >9.0), where the Bohr effect is complete and the contribution of the Bohr protons (endothermic) is abolished. Hence, as the pH falls, the apparent ΔH of the HbA reaction becomes less and less exothermic owing to the increasing contribution of the Bohr protons, which cancels some of the heat released upon O_2 binding (Antonini and Brunori, 1971).

Due to the exothermic character of the oxygenation process, a significant increase of body temperature may result in a substantial decrease of oxygen loading at the level of the lungs and hence a decrease of the amount of oxygen released by hemoglobin at the level of the tissues, whose metabolic requirements may not be fully satisfied. This situation may be that of birds during flight, which is known to be a very energy-requiring physical activity, so that the metabolic rates of flying birds increase to more than eight times the resting rate (Lutz, 1980; Giardina et al., 1985). This implies that birds, during normal sustained flight, must be able to dissipate more than eight times as much heat as during rest in order not to be over-heated (Giardina et al., 1990). Considering this particular aspect, a peculiar feature of the hemoglobin from the water hen (*Gallinula chloropus*), a bird capable of prolonged flight, is the progressive increase of the exothermic character of oxygen binding as the proton concentration increases. This is shown in Figure 1 and is opposite to the behavior of human hemoglobin. In fact, in the case of water hen hemoglobin, the overall ΔH of oxygen binding is at a minimum (in absolute value) at alkaline pH and tends to be more exothermic as the pH drops, despite the increasing endothermic concentration of the Bohr protons. This unusual thermodynamic behavior could be of great significance from a physiological point of view because it could well be related to the necessity of the animal to remove the heat metabolically released and to cool the whole organism. Thus, during the activity linked to prolonged flight, at the muscular level we observe an increased demand of oxygen, a great production of heat due to the increased rate of the meta-

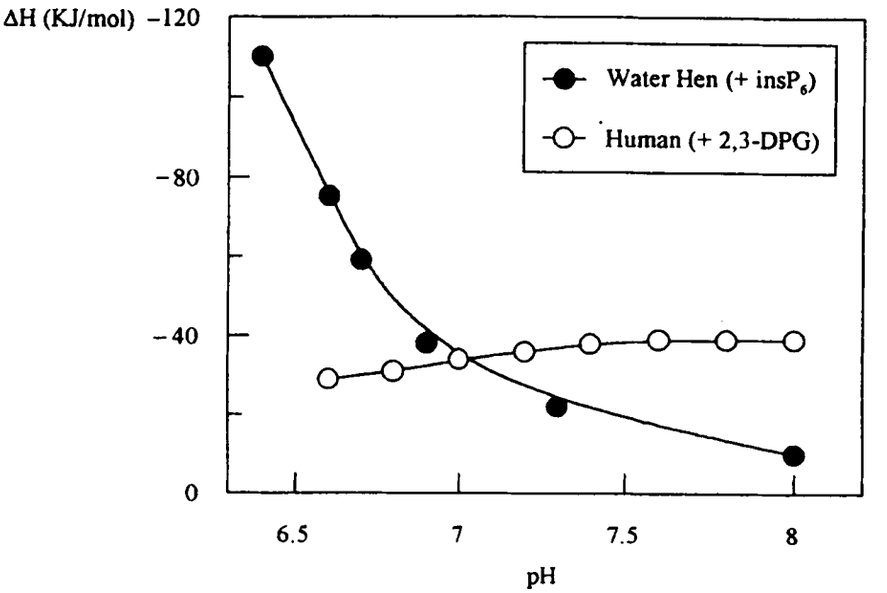

FIGURE 1. Overall heat of oxygenation of water hen (*Gallinula chloropus*) (closed circles) hemoglobin compared with that of adult human hemoglobin (open circles). The O_2 equilibria experiments were performed in 0.1 mol/l BisTris or Tris/HCl buffers plus 0.1 mol/l NaCl. Experiments on water hen Hb were performed in 3 mmol/l Ins-P_6, those on human Hb in 3 mmol/l 2,3-DPG. ΔH values were corrected for the heat contribution of O_2 in solution (−12.5 KJ/mol)

bolic reactions, and a contemporaneous decrease of pH brought about by lactic acid production and/or by the increase in temperature. Hemoglobin reaching the muscular tissue finds a more acid pH, which lowers its oxygen affinity and increases its ΔH of deoxygenation to maintain the body temperature at a reasonable level. In fact, assuming a pH value of approximately 6.6 at the level of the muscles, the hemoglobin from water hen would require, during the deoxygenation process, at least three times more heat than human hemoglobin, thereby contributing to a lowering of the amount of heat that has to be dissipated by other ways, such as evaporation of water and convection. Moreover, the very low ΔH of oxygen binding observed at alkaline pH values (three times lower with respect to that of human hemoglobin) could also be related to the necessity of the animal to optimize heat exchange at the level of the lungs.

Another interesting example that demonstrates the physiological role of the overall thermodynamic properties of the hemoglobin molecule is represented by human fetal hemoglobin. Here, the role of temperature and its interplay with heterotropic ligands, in particular 2,3-DPG, shows up very clearly. Thus, human fetal hemoglobin is known to display, at 20°C, an oxygen affinity lower than that of human adult hemoglobin when both proteins are in the absence of organic phosphates. The physiologically important reverse situation is achieved at 37°C after addition of 2,3-DPG, whose lower effect on fetal Hb is related to some amino acid substitutions present in γ-chains

(Giardina et al., 1993). In fact, the reduced binding of 2,3-DPG to fetal human hemoglobin has been explained by the replacement of a histidine residue at position 143 in β-chains with an uncharged serine in the γ-chains (Tomita, 1981).

This substitution results in the removal of two positive charges from the organic phosphate binding site, and explains the weaker binding of the anion 2,3-DPG to HbF. However, the difference in O_2 affinity observed at 37°C is not solely due to the different modulation power of 2,3-DPG with respect to adult and fetal hemoglobins. In fact, at 20°C after addition of 2,3-DPG, the lower effect of this organic phosphate on HbF renders almost identical the O_2 affinity of the two hemoglobins (Figure 2). It is only on going from 20 to 37°C that HbA shows a lower O_2 affinity, as it should be if oxygen has to be transferred from maternal to fetal blood. Hence, contrary to what is observed in the presence of only chloride (overall ΔH almost identical), the overall heat of oxygenation displayed by HbF in the presence of 2,3-DPG is significantly lower (~30%) than that of HbA. This is shown in Figure 3, in which the pH dependence of the overall ΔH is reported. As evident from the figure, while the ΔH of adult hemoglobin is slightly affected by protons, that of HbF is strongly affected by protons, so that at physiological pH (pH ≅ 7.4) it reaches a value of about –27 KJ/mol, or 30% lower with respect to adult hemoglobin. Although further investigations are needed in order to fully elucidate the molecular basis of the phenomenon, it is very clear that a body temperature

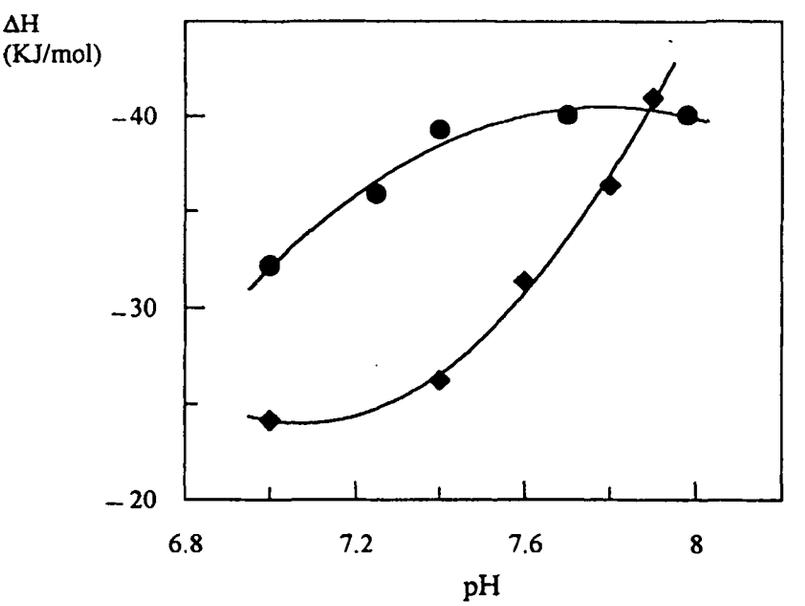

FIGURE 2. Overall ΔH of human (circles) and fetal (squares) hemoglobins. Equilibria conditions were 0.1 mol/l HEPES buffers plus 0.1 mol/l and 3 mmol/l 2,3-DPG. ΔH values were corrected for the heat contribution of O_2 in solution (–12.5 KJ/mol).

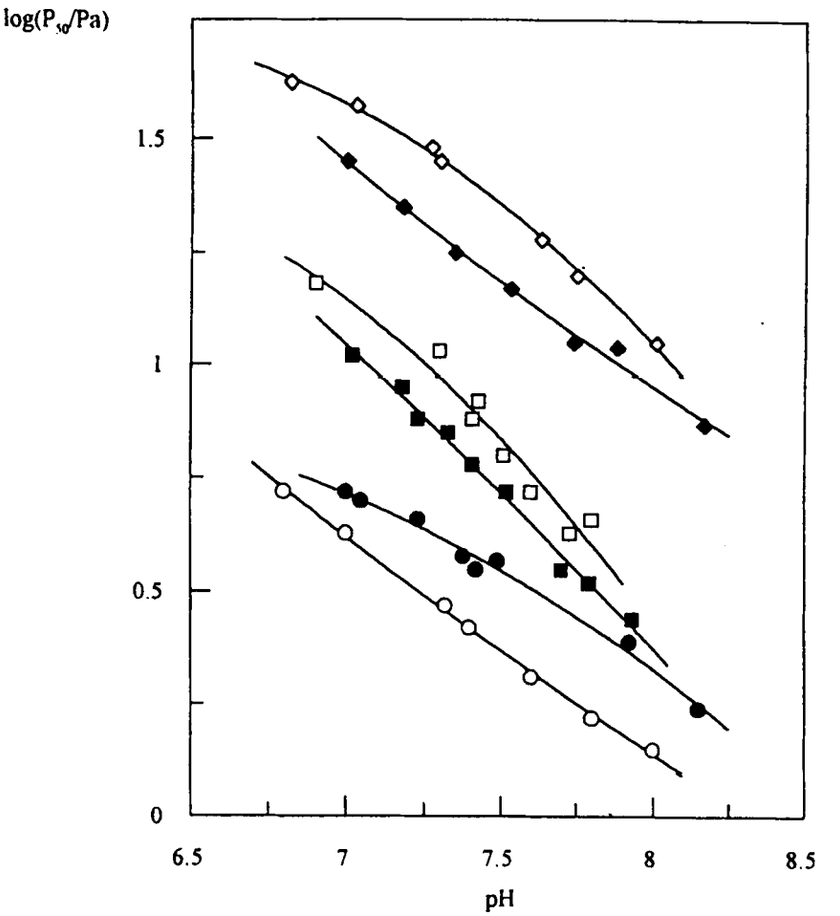

FIGURE 3. Effect of pH on the oxygen affinity of human adult (open symbols) and human fetal (closed symbols) hemoglobin. Circles refer to 0.1 mol/l HEPES plus 0.1 mol/l NaCl in the absence of 2,3-DPG at 20°C. Squares refer to the same condition but in the presence of 3 mmol/l 2,3-DPG. Rhombs refer to the previous conditions at 37°C.

of 37°C is essential in determining the extent of the difference in O_2 affinity between maternal and fetal blood and thus the amount of oxygen available for the fetus.

Apart from the gas exchange process, the reduced ΔH observed in fetal hemoglobin has additional physiological meanings because it may favor transfer of heat from the fetal to the maternal circulation on transfer of O_2 across the placenta, as more heat is absorbed on dissociation of oxygen from HbA than is released by O_2 binding to HbF. In other words, the placenta could be the place where O_2 and heat are exchanged in opposite directions (Figure 4). This point may have great relevance for maintaining constant the temperature of the well-insulated fetus (immersed in the amniotic fluid), contributing to the dissipation of the heat released by its metabolic activity.

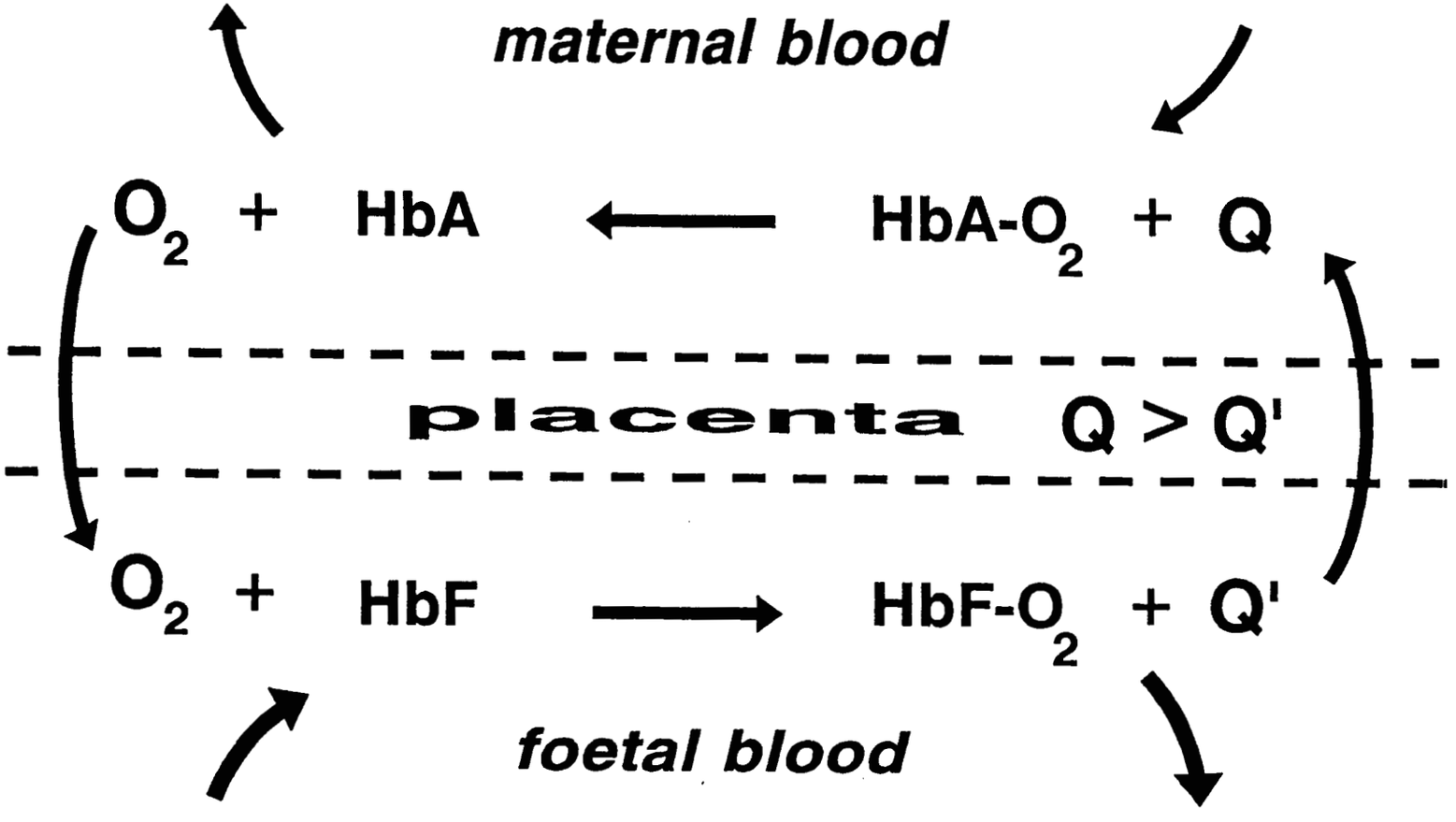

FIGURE 4. Flows of oxygen and heat associated with the oxygenation-deoxygenation cycle of adult and fetal hemoglobin at the level of the placenta.

IV. THE INTERACTION OF OXY- AND DEOXY-Hb WITH THE ERYTHROCYTE MEMBRANE AND CYTOSOLIC CONSTITUENTS

Very often, interesting phenomena based on intermolecular interactions, may be missed when a biological molecule is considered per se without taking into account the cellular scenery in which it performs its biological function. This could be the case of hemoglobin, which *in vivo* (i.e., in the erythrocytic milieu) exists under three different forms represented by oxygenated, deoxygenated, and partially oxidized molecules that display specific interactions with other cellular components. The first two derivatives (oxygenated and deoxygenated hemoglobin), whose relative proportions are continuously changing during the oxygenation-deoxygenation cycle, are known to interact reversibly and preferentially with several membrane proteins. Although the physiological significance of these reversible interactions is not fully established, some hypotheses of their role at the level of cellular metabolism are worth mentioning for future investigations.

Early experimental evidence has outlined the existence of a significant binding of ferrous hemoglobin to the cytoplasmic surface of the erythrocyte ghost membrane (Jacob et al., 1968), indicating a principal interaction with band 3 protein (Sayare and Fikiet, 1981). Band 3 is a transmembrane protein (≅ 93 kDa) that accounts for about 25% of the total red cell membrane proteins present, or about 1.2×10^6 copies per cell (Fairbanks et al., 1971). Its active form consists of a dimer with a membrane-spanning domain (≅ 50 kDa) that is responsible for anion exchange across the membrane and a cytoplasmic domain (≅ 43 kDa) that should exert modulatory functions (Jay and Cantley, 1986). Recently, a third subdomain has been proposed that is probably a flexible linker between the two principal domains (Wang et al., 1993). The cytoplasmic domain of band 3 is known to bind hemoglobin as well as cytoskeletal proteins (band 2.1 protein or ankyrin) (Bennett, 1985) and several glycolytic enzymes (Low, 1986) such as aldolase (Murthy et al., 1981), phosphofructokinase (Higashi et al., 1979), glyceraldehyde-3-phosphate dehydrogenase (Kliman and Steck, 1980), and lactate dehydrogenase (Harris and Winzor, 1990).

In this respect, it is important to remember that the binding of these glycolytic enzymes to band 3 generally inhibits, or at least greatly reduces, their catalytic activity (Low et al., 1993).

The amino-terminal fragment 1-23 of CDB3 seems to be the minimal structural requirement for Hb binding (Murthy et al., 1984). Moreover, an elegant study performed with a synthetic undecapeptide related to the end terminal of CDB3 (Walder et al., 1984) demonstrated that deoxy-Hb binds to band 3 via the amino terminus of the β-chain. In fact, the oxygen affinity of Hb is sensibly lowered in the presence of this synthetic fragment. The measured dissociation constant for deoxy-Hb was on the order of 0.3 mmol at pH 7.2 and 0.1 mol/l NaCl. Furthermore, the interaction with the red cell membrane is reduced for β-cross-linked Hb (Walder et al., 1984) and for HbA_{1c} (Cársky et al., 1985). Walder et al. (1984) suggested that the central cavity between the β-chains and the 2,3-DPG binding site are the regions involved in the interaction.

Recently, a study by Salhany and Cassoly (1989) confirmed that deoxy-Hb shows a higher affinity toward CDB3 with respect to oxy-Hb. These authors suggested that Hb may act as a heterotropic allosteric

effector on the anion transport activity of band 3 through modulation of its quaternary structure.

On the whole, CDB3, through its multiple binding with Hb and glycolytic enzymes, seems to be an erythrocyte membrane site that plays a central role in the metabolic traffic within the erythrocyte. At the moment, even though from the mosaic of information an accurate picture of the regulation pathways is not possible, some proposals can be made.

After its load within the red cell, glucose follows the main glycolytic pathway, which must fulfill two principal requirements: ATP and 2,3-DPG production (the latter produced by the well-known cycle of Rapoport and Luebering). Another essential glucose metabolic branch is represented by the shunt of pentose-phosphates, which is devoted to the production of a sufficient level of NADPH, necessary for the reduction of met-Hb (through met-Hb reductases, which are NADH- and NADPH-dependent systems) and for cell protection toward the intraerythrocytic production of superoxide anion and hydrogen peroxide (through the glutathione-peroxidase, superoxide dismutase, and catalase systems).

It is very unlikely that the relative activity of these two pathways is not modulated by the different physiological conditions in which the erythrocytes have to fulfill their functional role. In this respect, we may consider the erythrocytes, during their physiological function, as oscillating between two limit states: (1) a high-oxygenation state, just after pulmonary loading (arterial blood), and (2) a low-oxygenation state, after oxygen delivery at the level of tissues (venous blood). These two states may be thought to correspond, at a supramolecular level, to the quaternary R and T Hb states, respectively. Of course, within this picture, erythrocytes may move through infinite intermediate states, each of which is represented by a specific value of the ratio between the relative concentrations of oxy- and deoxy-Hb.

During the high-oxygenation state, the risk of oxidative stress should address erythrocyte metabolism toward the activation of those metabolic pathways that may lead to an increase of cell protection, namely, the pentose-phosphate shunt. During the low-oxygenation state, an increased production of ATP and 2,3-DPG could derive from the decrease in their free concentrations brought about by the well-known preferential binding of these molecules to deoxyhemoglobin.

Within this overall scheme, the interaction of Hb with the cytoplasmic domain of band 3 may display an important role in cellular metabolic regulation by linking red cell glycolysis to oxygen transport. Thus, at high oxygenation levels being the affinity of the Hb oxy form for CDB3 low, the binding of the glycolytic enzymes would be greatly favored and therefore the glycolytic pathway strongly inhibited. Hence, under these conditions, glucose-6-phosphate would be switched toward the pentose-phosphate shunt (Figure 5A). On the other hand, at low oxygenation levels, the high affinity of deoxy-Hb for CDB3 provides a cytoplasmatic release of the interacting glycolytic enzymes, which in turn results in activation of the main glycolytic pathway (Figure 5B). The average maximal stimulation of glycolysis observed after enzyme displacement from the polypeptide domain is near two thirds of the total (Low et al., 1993). This fraction of latent glycolytic activity in the cell corresponds closely with the fraction of glyceraldehyde-3-phosphate-dehydrogenase (65%) (Kliman and Steck, 1980), aldolase (40%) (Strapazon and Steck, 1976), and phosphofructokinase (50%) (Jenkins et al., 1985) estimated to be membrane bound *in vivo*.

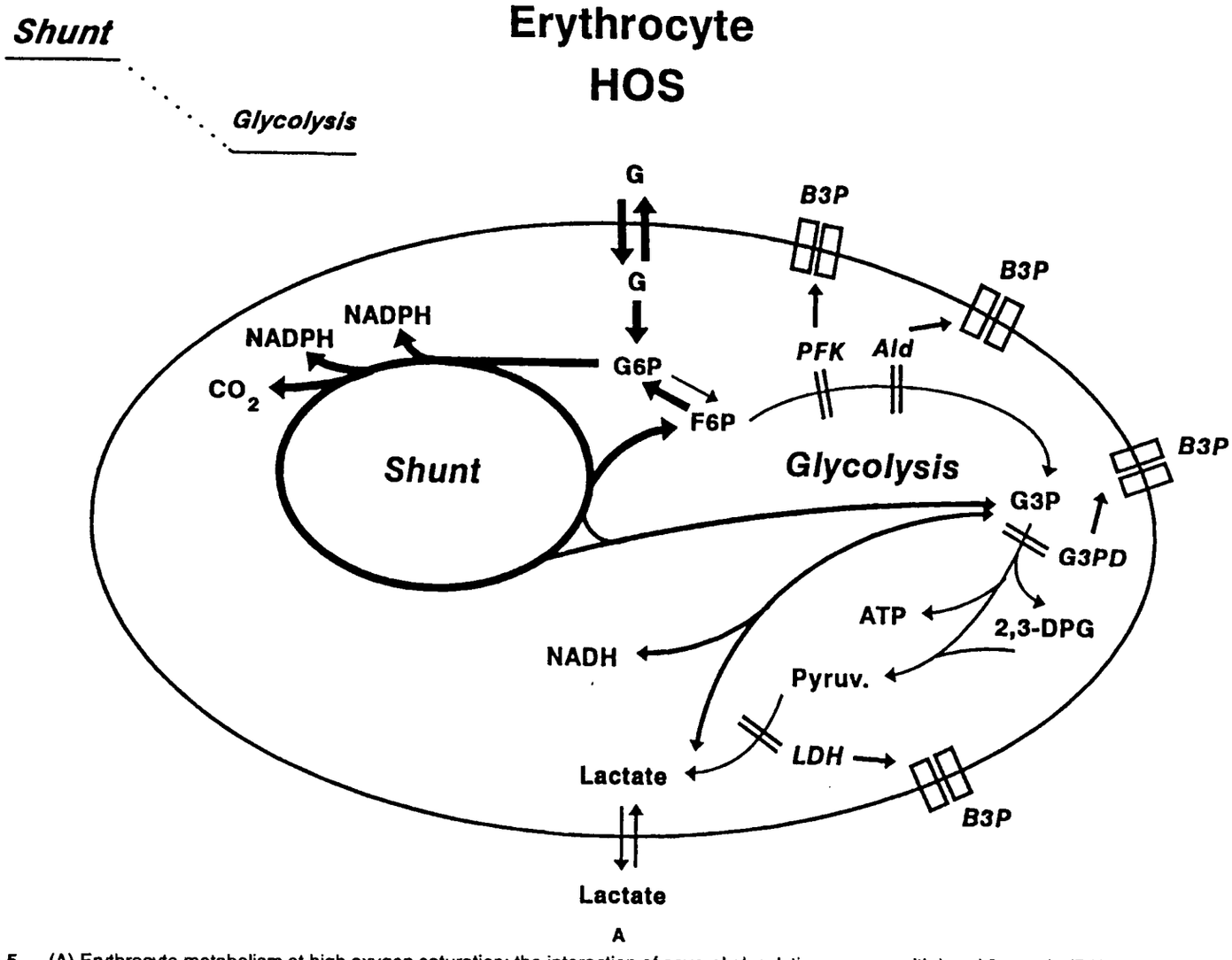

FIGURE 5. (A) Erythrocyte metabolism at high oxygen saturation; the interaction of several glycolytic enzymes with band 3 protein (B3P) reduces the glycolysis activity with a concomitant increase of the pentose shunt. (B) erythrocyte metabolism at low oxygen saturation; the interaction of deoxy-Hb with band 3 protein (B3P) releases the glycolytic enzymes, with an increase of the glycolysis and a concomitant decrease of the pentose shunt.

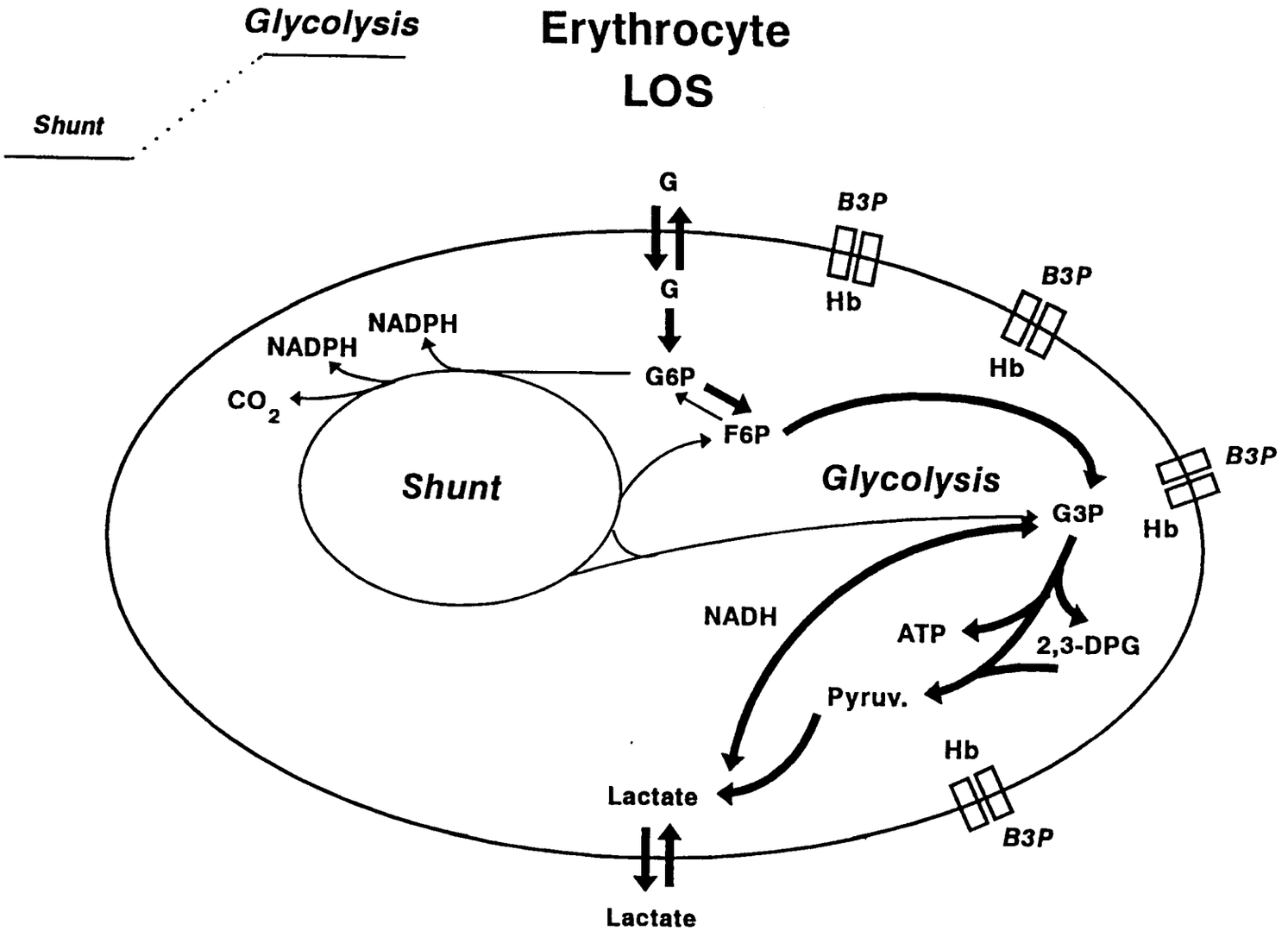

FIGURE 5B

Much experimental evidence is in agreement with this view. In particular, the early studies of Hamasaki et al. (1970) and Rapoport et al. (1976) reported an activation of glycolysis upon erythrocyte deoxygenation, indicating an important increase of phosphofructokinase activity (about 40%). As far as this enzyme is concerned, its interaction with CDB3 has been well documented. Higashi et al. (1979) found a high association constant (2×10^7 *M*) and indicated that ATP favors binding, while ADP and 2,3-DPG do not. In a very recent and elegant study, Low et al. (1993), through use of the Fab fragment of an antibody raised against residues 1–15 of band 3 (i.e., against the putative binding site of the glycolytic enzymes) reported a threefold increase in the rate of lactate production, whereas the load of erythrocyte cytoplasm with the peptide 1–15 deriving from band 3 protein resulted in a more than tenfold decrease of lactate production. The ability of the antibody to accelerate glycolysis has been ascribed to its capacity to block enzyme binding to the inhibitory site at the N terminus of band 3.

Moreover, we have to consider that the interchange of the erythrocyte between two quite different environments was already evidenced (Mohandas et al., 1983; Chasis and Mohandas, 1986), but it was attributed to different mechanical demands of the membrane, that is, a state of high membrane stability and a state with a high degree of deformability. These observations strongly indicated that the mechanical properties of the erythrocyte membrane could be subjected to regulation. Also in this case, the role of the Hb oxygenation state could be important. In fact, Lebbar et al. (1987) clearly demonstrated that deoxy-Hb interacts through its 2,3-DPG binding site with actin and tubulin and postulated an effect on the mechanical properties of the membrane. According to this hypothesis, the interaction of HbA and HbS in different conditions in human erythrocyte ghosts was found to affect the echinocyte-discocyte transition, the extent of endocytosis, the volume, and the sealing of ghosts (Wiedelman and Elbaum, 1983). Moreover, Devogel et al. (1977) established that the rate of deoxygenation of Hb is slower in the presence of spectrin and that spectrin increases Hb affinity for O_2, suggesting a significant interaction between the two proteins. All these considerations support the idea that the structure of the membrane is influenced by hemoglobin.

Also, the cytosolic environment, which has a relevant role in the control of erythrocyte structural and functional properties, appears to be under the influence of the allosteric characteristics of the hemoglobin molecule. As an example, we may consider the significant changes that occur in the free concentration of some intracellular constituents as a consequence of the oxygenation-deoxygenation cycle of hemoglobin. Thus the organic phosphates, present in highest concentration in the red cell, 2,3-DPG, and ATP, have been shown to bind specifically to deoxy-hemoglobin, resulting in a marked lowering of its oxygen affinity. The free concentration of magnesium (Mg^{2+}), nucleotides, and their respective complexes are, of course, influenced by the binding. Because magnesium-nucleotide complexes are substrates as well as modulators of hexokinase and phosphofructokinase, important controlling enzymes in the glycolytic sequence, an understanding of the modulation mechanism of the glycolytic chain requires a knowledge of the concentrations of these complexes available to the enzymes within the cell (Bunn et al., 1971; Berger et al., 1973; Achilles et al., 1981; Mairbaurl et al., 1993; Mota de Freitas et al., 1994). Hence, a detailed characterization may not omit the

interactions that exist between Hb and 2,3-DPG, Hb and ATP, Hb and MgATP, Hb and Cl^-, Mg^{2+} and 2,3-DPG, and Mg^{2+} and ATP (Table 1). The cytosolic milieu, in terms of cations and anion-free concentration, is therefore under the strong influence of the oxygenation state of the hemoglobin molecule, which appears to be the real controller of the metabolic status of the cell. In this respect, phosphofructokinase exemplifies the degree of complexity and integration that may characterize the erythrocyte function. This enzyme, in fact, is finely modulated, being activated by a number of metabolites and inhibited by ATP, 2,3-DPG, and protons. It also requires the presence of magnesium ions. At the intracellular level, when the erythrocyte releases oxygen, deoxygenated hemoglobin binds to band 3, 2,3-DPG, and ATP. As described above, the binding of deoxy-Hb to band 3 forces this protein to release phosphofructokinase into the cytoplasmatic milieu. At this level, phosphofructokinase may elicit its biological function because hemoglobin (1) buffers the increase of proton concentration via its Bohr effect, (2) decreases the free concentration of both 2,3-DPG and ATP, thereby removing their inhibitory effect, and (3) increases the free concentration of magnesium ions, which, upon the binding of 2,3-DPG and ATP to Hb, are released from their nucleotide complexes.

On the whole, the emerging scheme indicates that the energy involved in the oxygen-linked conformational transition of hemoglobin is utilized, within the cell, to modulate a number of other important cellular functions.

V. THE INTERACTION OF MET-Hb AND HEMICHROMES WITH THE ERYTHROCYTE MEMBRANE AND THEIR IMPLICATIONS IN THE AGING PROCESS

Red blood cells are especially vulnerable to oxidative injury because their mem-

Table 1
Distribution of Free and Bound Organic Phosphates and Magnesium in Oxygenated and Deoxygenated Erythrocytes (Bunn et al., 1971)

	Concentration (mmol/l erythrocytes)	
	Oxygenated	**Deoxygenated**
ATP total	2.00	2.00
ATP free	0.37	0.08
Mg/ATP	1.63	1.21
Hb/ATP	0.00	0.71
2,3-DPG total	7.20	7.20
2,3-DPG free	5.90	0.53
Mg/2,3-DPG	1.30	0.39
Hb/2,3-DPG	0.00	6.29
Mg^{2+} total	3.50	3.50
Mg^{2+} free	0.57	1.90

branes contain a variety of proteins that are susceptible to oxidative cross-linking and are embedded in phospholipids that are rich in unsaturated fatty acids. An abnormal association of met-Hb and derivatives with the membrane thus would be particularly harmful to the cell, targeting damage to membrane components.

The relationship between met-hemoglobin and the red cell membrane, investigated for more than 3 decades, has recently become a center of great attention, probably due to the potential implications in understanding the mechanism of various membrane pathologies. Of course, much of the renewed interest in the cytosol-membrane interface focuses on the role that met-Hb may play in the initiation and development of lipid peroxidation after coming into contact with the membrane lipids.

Although about 3% of the total intraerythrocytic hemoglobin is cycled each day to met-Hb (fully or partially oxidized hemoglobin), this derivative is normally present in the erythrocyte at a steady-state level of about 1%. When the ferrous (Fe^{2+}) iron of the heme is oxidized to Fe^{3+}, the Hb function is drastically modified so that an increased percentage of oxidation is accompanied by an increase of oxygen affinity and a decrease of cooperativity, up to the complete loss of the ability of exchanging the oxygen molecule (Antonini et al., 1965; Perrella et al., 1993). In this respect, the erythrocyte has developed a very efficient system devoted to the reduction of met-Hb and which, as described in the previous section, is connected to the pentose shunt. Furthermore, as hemoglobin begins to denature, minor structural rearrangements can lead to the formation of hemichrome, the H form, that can revert back to met-Hb or denature further toward the formation of irreversible hemichromes (B, C, and P types), which can aggregate to form Heinz bodies (Rachmilewitz et al., 1971).

On the whole, erythrocytes continuously fight against the risk of Hb oxidation until their defenses are quite exhausted. After about 120 d of permanence in the organism, the aged and damaged red cells must be selectively removed from the blood. Autoantibodies present in human serum selectively bind to senescent cells and induce their removal by phagocytes and macrophages (Schluter and Drenckhahn, 1986). The senescence antigen of red cells is not completely defined, but it seems to involve the outer domain of band 3 protein (Low, 1991), which in the aged cell appears to be modified in a way that generates a molecular mark of aged erythrocyte. Even though this modification is not completely understood at the molecular level, once again, the hemoglobin molecule, through its oxidation and denaturation, seems to be the primary cause of the antigen exposure (Giardina et al., 1991).

In this respect, Low et al. (1985) have indicated that one of the mechanisms at the basis of the removal of aged erythrocytes starts from the association of hemichromes with the cytoplasmic domain of band 3 protein. This interaction, after a single bimolecular complex, propagates with a 2.5:1 stoichiometry into tightly aggregate copolymers (Waugh and Low, 1985). The clustering of band 3, which derives from this aggregation, provides the recognition site for the binding of autoantibodies to senescent cells. In this respect, it may be worthwhile to recall that treatment of erythrocytes with phenyl hydrazine leads to an increase of the binding of autologous IgG that is one to three orders of magnitude higher with respect to untreated erythrocytes. Moreover, immunofluorescence experiments showed that the clustering of B3P is the recognition site of the autologous IgG.

Furthermore, the use of a monoclonal antibody directed against B3P revealed, through immunoblotting techniques, an increase of the binding in erythrocytes from patients affected by various red cell pathologies, such as G6PD and PK deficiency, indicating that band 3 protein is very sensitive to cellular alterations (Giuliani et al., 1993). The whole body of data suggests that several pathological erythrocyte states might promote the premature appearance of the senescent antigen at the level of the B3P.

Because the first step in the generation of hemichromes is the formation of met-Hb, elucidation of the interaction of partially oxidized Hb with the membrane has to be considered. Giardina et al. (1991) have established that about 50% of the oxidized Hb bound to the membrane is probably in the form of hemichromes, while about 50% is constituted by various oxidation intermediates of Hb (i.e., met-Hb). However, this ratio is strongly dependent on the age of the cell and/or on the specific erythrocyte pathology (e.g., G6PD deficiency, presence of HbS, etc.). The same study established that partially oxidized hemoglobin may be preferentially bound by the erythrocytic membrane even in the presence of oxygenated Hb. This binding has been shown to be abolished, or at least greatly decreased, by the addition of ligands of the ferric form of Hb, such as azide, cyanide, or OH^-. Experiments performed by using the oxidized isolated α- and β-chains suggest, with respect to the preferential interaction described above, a major role of the α-chains. This observation is in agreement with an oxidation rate of the α-chains much faster than that of the β-chains (Mansouri and Winterhalter, 1974). For this reason, the intermediate oxidized Hb *in vivo* should be present mainly as $\alpha_2^{+3}\beta_2^{+2}$ rather than the $\alpha_2^{+2}\beta_2^{+3}$ valency hybrid (Tomoda et al., 1978). It may be relevant to remember that, in the case of the cross-links between spectrin and hemoglobin, it has been demonstrated that α-chains are ten times more reactive than the partner β-chains.

The specific interaction of met-Hb with the plasma membrane may be of great physiological significance in relation to the enzymatic reducing mechanisms that maintain the hemoglobin molecule in its active state. The plasma membrane may be thought to play an active role in the reduction of hemoglobin, at least because it may segregate the partial oxidation intermediates at the level of the appropriate membrane district.

In old or abnormal erythrocytes, where the reduction systems may be altered or not sufficiently active, met-Hb, instead of being readily reduced, can be slowly transformed into hemichromes that in turn may react with band 3 and/or spectrin, initiating some of the cross-linking processes that are known to occur *in vivo*. Within this overall scheme, we have to carefully consider that the reversible and preferential interaction of met-Hb with the plasma membrane is upstream of all the reactions that may lead to elimination of old and damaged erythrocytes.

VI. HEMOGLOBIN AND ITS POSSIBLE IMPLICATION IN GENETIC ADAPTATIONS TO MALARIA

We have been intrigued by the idea that the protection against malaria afforded by some genetic traits, such as HbS, HbE, hereditary persistence of fetal hemoglobin (HPFH), α- and β-thalassemia, and G6PD-deficiency, may be viewed as based on oxidative processes initialized by hemoglobin binding to the red blood cell membrane. A wealth of evidence supports the idea that

abnormal red blood cells are more susceptible to Hb-mediated oxidative damage than normal erythrocytes. In fact, they are characterized by a strong tendency toward increased "membrane-bound hemoglobin" and increased oxidative stress (Brunori et al., 1975; Hebbel, 1991). Moreover, HbS, HbF, and HbA_2 (elevated in β-thalassemic individuals) are known to autoxidize faster and to have a higher affinity for the red blood cell membrane compared with normal human hemoglobin A (Klipstein and Ranney, 1960; Hebbel et al., 1988). In addition, coclustering of band 3 with Heinz bodies and the formation of abnormal Hb-spectrin adducts may be observed in thalassemic and hemoglobinopathic red blood cells (Schluter et al., 1986; Fortier et al., 1988).

In particular, most of the membrane-bound hemoglobin-mediated cellular perturbations involved in normal red blood cell senescence were first demonstrated in the case of HbS (i.e., in sickle cells). The hemoglobin/membrane interaction and the following oxidant stress targeted to the membrane are in fact claimed as an important factor in sickle disease pathophysiology, either by directly promoting membrane damage or by facilitating sickling itself (Hebbel, 1991).

Of course, the situation may display a different degree of complexity, depending on the given genetic pathology under investigation, as exemplified by the cases of G6PD deficiency and of HbS. In the former case, it has been elegantly shown (Usanga and Luzzatto, 1985) that when *Plasmodium falciparum* is transferred serially throughout G6PD-deficient red cells, it undergoes adaptive changes that gradually improve its ability to multiply in these deficient cells through its own production of G6PD, implying that it has a gene specifying this enzyme. This can be considered one of the first examples of an adaptive phenomenon occurring in a parasitic protozoon to a mutant host cell environment by virtue of the modulation of a specific parasite gene, which is switched on only when G6PD-deficient erythrocytes are used as host cells. On this basis, the observation that female heterozygotes for G6PD deficiency are protected from lethal malaria infections has been studied further. In fact, *P. falciparum* merozoites, emerging from normal erythrocytes, have, on average, an even chance of infecting a normal or a G6PD-deficient cell, implying a 50% reduction of the chance of completing successfully the next schizogonic cycle. In this particular case, therefore, the stimulus to the parasite to adapt by G6PD gene activation is significantly attenuated (Bienzle et al., 1979). Male hemizygotes completely deficient in G6PD may have a smaller, but perhaps significant advantage. On the whole, retarded growth of the parasite of even three to five cycles (6 to 10 d) during adaptation by G6PD gene activation may decrease morbidity and mortality.

However, it has been clearly shown that, although the parasite does possess additional pathways (induced G6PD and glutamate pathway) for the generation of NADPH that may serve its own metabolic needs, the host cell, and hence the parasite itself, remains vulnerable to oxidant stress (Roth et al., 1986). This finding is in full agreement with experiments showing an inhibition of growth when an oxidative stress is applied to an *in vitro* culture of *P. falciparum*-G6PD-deficient cells (Friedman, 1979; Roth et al., 1986). In fact, the quantity of parasite-encoded enzyme that is induced is small compared with the activity found in normal, uninfected red blood cells. Hence, unadapted parasites growing in G6PD-deficient red blood cells are eight times more sensitive than parasites grown in normal red blood cells, and preadaptation by growth in G6PD-deficient cells increases resistance to oxida-

tive stress only modestly. Therefore, despite the ability of the parasite to switch on its own production of G6PD, oxidative stress remains a major hypothesis for G6PD deficiency-dependent mechanisms of malaria resistance.

In the case of HbS, the situation appears more complex because several mechanisms, whose relative weights are not easy to specify, may be contemporaneously involved. In this respect, it should be recalled that the rate of sickling of deoxygenated, parasitized red blood cells from β^s heterozygotes resulted two to eight times faster than nonparasitized cells in the same blood (Luzzatto et al., 1970). These and other data (Roth et al., 1978) were interpreted as suggesting that accelerated sickling of parasitized red blood cells might predispose such infected cells to early removal from the circulation, thereby limiting the overall level of parasitemia. Moreover, polymerized hemoglobin appears to be a poor substrate for the protease of *P. falciparum,* and the HbS polymer may also interfere directly with some critical function of the parasite, which is apparently killed within sickled erythrocytes. All these mechanisms are consistent with the observation that sickle trait individuals are not resistant to becoming infected with the parasite but are less likely to die of their infection than individuals with normal hemoglobin. However, oxidative stresses cannot be disregarded, as demonstrated by the observation concerning the role of fetal hemoglobin in carriers of the β^s gene who exhibit a significant retardation in the fetal-to-adult switch during the first 5 years of life. In fact, *P. falciparum* growth is retarded in cord blood cells containing about 85% HbF, in red blood cells that contain 20% HbF obtained from infants, and in red blood cells containing 100% HbF from adult homozygotes for HPFH. Retardation of growth in all these cells strongly implicates the role of HbF in increasing oxidative stress, thereby accounting for retarded parasite growth (Nagel and Roth, 1989). On the whole, variant red blood cells seem to be more susceptible to hemoglobin-induced membrane damage than normal erythrocytes. Hence, the reduced lifespan of erythrocytes carrying the more severe syndromes could be explained, at least in part, by this mechanism.

Preliminary experiments on *P. falciparum* infection have shown that membrane-bound hemoglobin is significantly increased in *Plasmodium*-infected red blood cells, this being more evident in the presence of G6PD deficiency. The observed increase in membrane-bound hemoglobin suggests that in malaria-infected cells an oxidative damage is probably acting at the membrane level. Interestingly, some previously described properties of infected red blood cell ghosts may be explained on the basis of an increase of membrane-bound hemoglobin: (1) a markedly restricted mobility of the transmembrane domain of band 3 (Tilley et al., 1990) following cross-linking via hemicrome (McPherson et al., 1992) and (2) the higher susceptibility to oxidative stress of plasma membrane from parasitized erythrocytes depleted of their cytosol (Simoes et al., 1992).

On the whole, it seems that some red cell abnormalities and malaria infection may enhance the tendency of hemoglobin to bind the plasma membrane, therefore promoting oxidative damage at this level. Hence, it can be supposed that membrane-bound hemoglobin-mediated oxidative damage in variant red blood cells, further enhanced by malaria infection, could lead to early elimination of the infected cells, thereby protecting the whole organism with respect to the malaria parasite. Paradoxically, we may say that, with respect to *Plasmodium,* the strength of the erythrocytes is their weakness toward the oxidation processes.

VII. ENZYMATIC ACTIVITIES OF HEMOGLOBIN AND INTERACTIONS WITH DRUGS

During an investigation concerning the subcellular localization of aniline hydroxylase activity in human placenta (Juchau and Symms, 1972), it was observed that hemoglobin may exhibit the enzymatic activity characteristic of the monooxygenase function of the liver microsomal cytochrome P-450. Later on, studies on intact erythrocytes and hemolysates (Blisard and Mieyal, 1979) showed that the rate of the reaction is directly proportional to the concentration of oxyhemoglobin and that the catalytic activity is lost when the intraerythrocytic hemoglobin is fully oxidized to met-hemoglobin. On this basis, the monooxygenase activity has been thought to be displayed by the oxy derivative of hemoglobin. Moreover, NADPH stimulates the monooxygenase-like activity of human erythrocytes as long as an accessory electron carrier, such as methylene blue, is provided. This finding, together with the biphasic dependence on the NADPH concentration in the presence of methylene blue (Blisard and Mieyal, 1981), suggested a possible role of the NADPH-dependent met-hemoglobin reductase.

In addition, a study performed by using different substrates (Starke et al., 1984) evidenced that, in a reconstituted system including NADPH and cytochrome P-450 reductase from rat liver, hemoglobin may exhibit, besides the hydroxylation reaction, *N*- and *O*-demethylation activities that may be characterized by a distinct regioselectivity. For some substrates, this activity may also be displayed by intact erythrocytes, the relative activities being qualitatively similar to those observed with isolated hemoglobin.

As to the relative role of the α- and β-chains, it has been shown that fetal erythrocytes are three to five times more active than adult erythrocytes (Blisard and Mieyal, 1980), thereby suggesting a major role of the non-α-chains. In this respect, the use of different semihemoglobins (Hb in which one chain is heme deprived) and valence hybrids (Ferraiolo et al., 1984) confirmed that the monooxygenase activity primarily involves the β-subunits.

On the whole, these regioselective and broad monooxygenase-like activities of hemoglobin resemble those of an authentic monooxygenase enzyme such as cytochrome P-450. The general characteristics include utilization of NADPH and cytochrome P-450 reductase as electron transfer systems, inhibition of activity by carbon monoxide, hydrophobic heme environment, and oligomeric quaternary structure. Hence, these enzymatic activities of hemoglobin seem to derive from some basic structural similarities with other hemoproteins, and possess some fascinating evolutionary implications. In this respect, the unusual nonflavin character of the NADPH-dependent reductase of erythrocytes, when compared with other reductases, and the requirement of an exogenous electron-carrier cofactor, might be related to a limitation of the hydroxylase and demethylase activity of hemoglobin in favor and protection of its oxygen carrier function (Blisard and Mieyal, 1981).

Other side activities of Hb concern the oxidative condensation of aminophenols. Hence, differently substituted ortho- and paraaminophenols may be condensed to phenoxazinones (Eckert and Eyer, 1983; Tomoda et al., 1986, 1992), while 3-hydroxyanthranilic acid is rapidly transformed into cinnabarinic acid (Tomoda et al., 1984). These reactions appear to be catalyzed by either oxy-Hb or aquo met-Hb, while CO-Hb and cyano met-Hb are ineffective. On this basis, it was proposed (Tomoda et al., 1992) that

the reaction proceeds through a two-electron oxidation of aminophenol, therefore leading to a quinoneimine. In this case, the activity of hemoglobin is comparable to that observed in the oxidative condensation brought about by several copper-containing proteins such as microbial actinomycin synthase and tyrosinase, or by some hemoproteins such as catalase and the cytochrome c oxidase system.

In addition to all the aforementioned catalytic activities, we cannot disregard the hydrolysis of aromatic esters (Elbaum and Nagel, 1981) brought about by oxy-Hb, which displays an esterase activity intermediate between those of bovine albumin and carbonic anhydrase. The inhibitory effect of 2,3-DPG and the pH dependence of the reaction are consistent with the participation of a histidine residue at the level of the organic phosphate binding site (probably His-β2). Actually, it is difficult to establish if the Hb esterase activity is of physiological relevance, being an effective physiological substrate unknown and not easily postulable. Although the esterase activity is low compared with that reported for other proteins, such as carbonic anhydrase or α-chymotrypsin, we have to consider that it could be compensated for by the very high intraerythrocytic protein concentration (Elbaum and Nagel, 1981).

Finally, we have to consider that free hemoglobin, like other iron-rich substances, may originate a hydroxyl-radical in the presence of a superoxide anion-generating system in a dose-dependent fashion (Sadrzadeh et al., 1984), a reaction characteristic of peroxidases. As in the case of the monooxygenase activity, the oxidation of Hb switches off this peroxidase activity, which is also greatly decreased by carbon monoxide or the addition of catalase. This activity is also suppressed by haptoglobin via an unknown mechanism (Sadrzadeh et al., 1984), suggesting that the peroxidase activity of free hemoglobin is potentially hazardous. For this reason, the process is thought to proceed via the reaction between ferrous heme and hydrogen peroxide. Moreover, a modulation mechanism linked to structural changes of the protein moiety can be postulated, as the activity is inhibited by glycation of the protein (Khoo et al., 1984).

The described enzymatic activities of the hemoglobin molecule may be considered as some side reactions deriving from the similarity of the Hb structure with other heme-bearing proteins or from the intrinsic properties of the iron atom. Although they could not have a particular physiological significance, their role in some pathological processes such as hemolysis and ischemia has to be carefully considered because their full achievement could lead to deleterious and dangerous molecular and cellular alterations. In this respect, the encapsulation of Hb within the erythrocyte, the specific requirements of cofactors normally lacking at the intracellular level, and the specific binding of haptoglobin to free Hb are all elements that seem directed more toward a general inhibition of these alternative functions than toward their exploitation.

These Hb activities should also be carefully considered in case of drug administration, as many drugs may act as anomalous substrates, inducing massive hemoglobin oxidation and a molecular cascade toward premature hemolysis. Hence, these enzymatic activities could be a guideline for understanding the hematological toxic effects of several compounds. In this respect, it is particularly interesting that methylene blue, a possible electron carrier in Hb monooxygenase activity (Blisard and Mieyal, 1981), as well as many drugs that possess an aromatic ring activated toward

electrophilic aromatic substitution (i.e., nalidixic acid, doxorubicine, and phenazopyridine), are substances that cannot be administered in patients affected by G6PD deficiency. It is on this basis that the use of chloroquine, whose aromatic rings are deactivated by an alogen substitution, is allowed, while that of primaquine, which possesses an aromatic ring activated by a methoxy substituent, is not advisable. It may be worthwhile to recall that chloroquine is used as an antimalarial drug whose mechanism of action is based on its interference with malaria pigment (hemozoin) production, leading to a toxic accumulation of ferriprotoporphyrin IX (Orjih and Fitch, 1993). Chloroquine, in fact, inhibits the activity of heme polymerase, an enzyme that catalyzes the polymerization of ferriprotoporphirin IX to form β-hematin, the principal pigment of hemozoin. The emergence of strains of *P. falciparum* malaria parasites resistant to chloroquine and quinine have led to the development of several new antimalarials, among which mefloquine is the most promising. In fact, because mefloquine is accumulated by red cells, single doses of the drug offer prophylaxis for up to 3 weeks and long-term chemotherapeutic effects (San George et al., 1984).

Mefloquine serves as an example of the role that hemoglobin may display in a given pharmacological treatment. An elegant study using ^{19}F-NMR (San George et al., 1984) demonstrated that mefloquine interacts with hemoglobin through a single binding site (per tetramer) with an affinity constant of 10^3. Mefloquine accumulates in the red cell with a red cell-to-suspending medium distribution ratio that ranges between 15 and 60, depending on the extraerythrocytic concentration of the drug. The binding involves both the membrane and the hemoglobin molecule. Initially, mefloquine is taken up by red cells due to high-affinity binding sites on the red cell membrane. As these sites become saturated, binding to lower-affinity membrane sites takes place. At this point, the drug crosses the red cell membrane and binds to the intraerythrocytic hemoglobin, freely exchanging between these two different sites. Exchange of the membrane-bound mefloquine with intracellular hemoglobin, besides being at the basis of its very low release from the cell, provides a mechanism by which the drug becomes accessible to the parasite, where its antimalarial activity shows up. However, it is a pity that a characterization of the effect of mefloquine on the functional properties of hemoglobin is lacking. Thus, on the basis of what has been reported above, iatrogenic modifications of the functional properties of red blood cells have to be considered as a real possibility. This should not be viewed only as a negative side effect because it may also allow new therapeutic approaches in different diseases. In some cases, such as that of fibrates (clofibric acid, bezafibrate, and gemfibrozil), this possibility has been verified. The impressive effect of fibrates on the oxygen affinity of intraerythrocytic hemoglobin has been studied, in fact, as a possible tool (1) for hindering *in vivo* polymerization of HbS and (2) to improve oxygenation of the neoplastic mass, thereby sensitizing it toward the action of radiotherapy (Siemann et al., 1989; Hirst and Wood, 1991). Unfortunately, in both cases, the concentration necessary to have the specific activity was greater than that usually allowed by the drug toxicity (Scatena et al., 1995). However, the possibility that drugs interacting with hemoglobin may modify the functional properties of red blood cells has been generally underestimated and should be carefully examined in the pharmacological approach to new and old therapeutic agents.

VIII. THE DESTINY OF HEMOGLOBIN CATABOLITES

When aged or damaged erythrocytes are removed from the bloodstream, hemoglobin is subjected to a series of degradation processes that should carry out its elimination from the organism. During these processes, in line with the principle of the minimum waste of energy, some catabolites could be utilized for modulating other biochemical pathways. That this is the case is clearly indicated by several recent papers showing that peptides deriving from the apoprotein may still have a physiological role.

During the purification process of cytochrophin-4 from bovine blood, an additional peptide with opioid-like activity has been recognized as the fragment 34–37 of the β-chain of bovine hemoglobin, which in turn corresponds to fragment 35–38 in β, γ, δ, and ε human hemoglobin chains. A similar opioid-like activity also is shown by the pentapeptide 35–39 of the same human chains (corresponding to residues 34–38 of bovine β-chains). These two peptides were called hemorphin-4 and -5, respectively (Brantl et al., 1986). On the basis of our present knowledge, it cannot be excluded that anodyn, a small uncharacterized peptide from human blood showing high affinity for opiate receptors (Pert et al., 1976), may be coincident with one of these two hemorphins.

Subsequently, a naloxone-reversible opioid action of hemorphins *in vivo* was observed (Davis et al., 1989). Because a significant number of red blood cells are metabolized under normal physiological conditions and the half-life of hemorphins is in the range of 60 min, a physiological role of these blood-borne peptides is very likely.

Recently, Piot et al. (1992) compared different amino acid sequences obtained by peptic hydrolysis of bovine hemoglobin and found various peptides with high opioid activity. These peptides originate from the same region of the β-chain (residues 32–41 of human Hb and 31–40 of bovine Hb), probably by enzymatic digestion at the level of the pituitary gland. It could be concluded that this part of the protein is a domain with a potential endorphin activity that may appear during protein degradation.

Moreover, recent data suggest that hemorphins might display multiple regulatory roles; Lantz et al. (1991) reported that LVV-hemorphin-6, found in large amounts in the pituitary gland and having a high stability toward proteolytic enzyme activity, also acts as an angiotensin-converting enzyme inhibitor. Hence, even though the concentration of hemorphins in the circulation has not yet been determined, the latter example underscores the importance these peptides may have for the interconnection of apparently unrelated physiological processes and their regulatory mechanisms.

Along the same line, protease digestion of bovine hemoglobin gives rise to a fragment (residues 129–134) of the α-chains that has been shown to display bradykinin-potentiating activity and hence a possible regulatory role with regard to blood pressure and/or the coagulative cascade. As shown in Table 2, the corresponding human fragment differs in only one residue, a serine instead of an asparagine.

Table 2 reports the sequence of various hemoglobin-derived peptides endowed with biological activities in comparison with that of β-casomorphin-4 and -5 and cytochrophin-4 and -5.

Another catabolic product of hemoglobin is carbon monoxide. It is produced during the transformation of heme to biliver-

Table 2
Sequences of Biological Active Peptides Related to Hemoglobin

	Bovine Hb β-Chain 31.....40 LVVYPWTQRF	Human Hb β-Chain 32.....41 LVVYPWTQRF
Hemorphin-4	YPWT	YPWT
Hemorphin-5	YPWTQ	YPWTQ
Hemorphin-6		YPWTQR
LVV-Hemorphin-6		LVVYPWTQR
VV-Hemorphin-7	LVVYPWTQR	
Cytochrophin-4	YPFT	
Cytochrophin-5	YPFTI	
β-Casomorphyn-4	YPFP	YPFV
β-Casomorphyn-5	YPFPG	
	Hb α-Chain 129.....134 LANVST	**Hb α-Chain 129.....134 LASVST**

din, through the catalysis of heme oxygenase. Until recently, carbon monoxide has been considered only as a poison due to its high-affinity binding for cytochromes, cytochrome oxidase, and hemoglobin and the consequent impairment of their function. Nevertheless, several experimental results led to the hypothesis that small endogenous concentrations of CO may play a relevant physiological role. In fact, it has been suggested that endogenous CO modulates blood vessel tone through the activation of guanylyl cyclase (Vedernikov et al., 1989; Utz and Ullrich, 1991). This CO relaxing effect on coronary and aortic vascular smooth muscle (Ramos et al., 1989) seems to be related to a time-dependent and reversible increase in levels of cGMP (Lin and McGrath, 1988). Although a prostaglandin mechanism is believed to be responsible for the relaxation of the ductus venosus and arteriosus, the role of endogenous carbon monoxide cannot be disregarded (Marks et al., 1991). This consideration is based on the possible binding of CO to the heme moiety of cytosolic guanylyl cyclase and/or on the interaction with enzymatic Fe-S centers. Guanylyl cyclase (Brüne and Ullrich, 1987) also seems to be involved in the observed inhibition of platelet aggregation (Mansouri and Perry, 1982, 1983). Even though small quantities of endogenous CO are probably produced during lipid peroxidation, the principal biochemical pathway for CO endogenous production is related to heme-oxygenase, which is particularly abundant in organs characterized by a high presence of reticuloendothelial cells. In this respect, it may be worthwhile to recall that this enzyme displays two isoforms, one of which appears to be inducible by heme and is predominant in tissues such as spleen and liver, where heme catabolism normally takes place (Cruse and Maines, 1988).

A role has also been postulated for bilirubin, the end product of porphyrin catabolism in mammals (Stocker et al., 1987). In fact, bilirubin scavenges peroxyl radicals

in either homogeneous solution or multilamellar liposomes. The antioxidant activity observed in liposomes is higher than that of α-tocopherol, which is regarded as the best antioxidant of lipid peroxidation. These findings support the idea of a "beneficial" activity as a chain-breaking antioxidant. In this view, pathologies or acute stress characterized by an increased hemolysis might generate a high quantity of circulating bilirubin (free or protein bound), that can help the normal antioxidant pool of the organism to prevent the deleterious effect of high iron levels.

IX. CONCLUSIONS

In light of the considerations reported here, the hemoglobin molecule appears, once again, as a stimulating model for understanding the different structural and functional molecular mechanisms that are at the basis of the different levels of biological organization of a given organism. Hence, a specific molecule is subjected, as far as its main function is concerned, to modulation mechanisms that optimize its functional behavior to the specific physiological requirements. These mechanisms are, however, much more sophisticated than what may be thought looking only at the basic function. In fact, some of them may be utilized at the cellular level to perform a number of other functions that are not minor, in general terms, with respect to the basic function of the considered macromolecule. As an example, we may take the interaction of hemoglobin with band 3, which appears of great importance with respect to ion traffic within the cell and the regulation of glycolysis in dependence of the intracellular oxygen concentration. In this particular case, the energy involved in the quaternary conformational transition that accompanies ligand binding is exploited to modulate both the function of band 3 and the glycolytic pathway. Moreover, the probable involvement of hemoglobin in the process of heat exchange, in both birds and the human fetus, provides a strong link between oxygen transport and the more general physiological requirements, which are only apparently far from the basic function of hemoglobin.

Along the same line, we may consider the role of the hemoglobin molecule in the production of carbon monoxide and hemorphins, which more or less directly correlates this molecule with other metabolic pathways that are of great importance in the economy of the whole organism.

From this point of view, a given molecule should be considered as one piece of a multidimensional structural and functional mosaic in continuous interaction with a number of other biological systems. The complexity of the picture is enhanced by the time dependence of these interactions and the interrelationships with the life span of the molecule and its degradation products.

On the whole, the emerging picture indicates a complex organization of the information contained in the Hb molecule, which is highly coordinated with other proteins, enzymes, and organs, first in the interior of the erythrocyte and later in the transmission of information to other organism districts during its catabolism. Finally, it is suggestive that the exon structure of globin genes has been interpreted as a mosaic of ancestral phenotypic functions, which organized themselves to fulfill a more articulated and complex molecular task (Blake, 1983; Eaton, 1980). This may be viewed as the first part of the mosaic organization of a given biological system that extends from the intramolecular to the intermolecular level.

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